Development of Sex Cord Stromal Tumors after Heterotopic Transplantation of Cryopreserved Ovarian Tissue in Rats

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Abstract. *Chemoradiotherapy has substantially improved life expectancy in young women with cancer, but these treatments often cause infertility. One method of preserving fertility is to cryopreserve ovarian tissue, with subsequent autotransplantation of the tissue after successful anticancer therapy. This study examined the long-term effect of heterotopic transplantation of cryopreserved ovarian tissue on the histology. Ovarian tissue from rats was cryopreserved using a slow-freezing protocol. After thawing, the tissue pieces were transplanted under the splenic capsule in 14 rats of the same inbred strain and remained there for 210 or 300 days. Sex cord stromal tumors, consisting mainly of granulosa cells, were found in all of the rats. Although the hormonal situation in rats cannot be directly compared to that in humans, the development of sex cord stromal tumors in this animal model may be worth considering when cryopreserved ovarian tissue is transplanted heterotopically in fertility-preserving programs for cancer patients.*

Aggressive chemotherapy and radiotherapy in young women with cancer have greatly enhanced life expectancy in these patients, but the treatments often cause infertility due to massive destruction of the ovarian reserve, resulting in premature ovarian failure. These young women have to face years of hormone replacement therapy, as well as the prospect of infertility, with the resulting additional psychological stress (1, 2).

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In male patients, semen cryopreservation is an effective and noninvasive technique that has already yielded thousands of successful pregnancies. For women, however, cryopreservation of oocytes is still difficult, with low rates of survival and development of the oocytes (3, 4).

Other options, also with limited applications, include ovarian transposition before radiation exposure and treatment with gonadotropin-releasing hormone (GnRH) analogs (5, 6). The most promising method, however, is cryopreservation of ovarian tissue before oncological treatments, because of the large number of follicles that survive the freezing/thawing procedure (7-9). The problem that arises after cryopreservation is how to use the frozen material to achieve a pregnancy. The majority of follicles that survive cryopreservation are primordial (10-13).

There are three ways of getting these follicles to develop to maturity. The first method is autografting: either orthotopic, which has recently yielded the first human pregnancy after cryopreservation of ovarian tissue (14); or heterotopic, for example subcutaneously, in the forearm (15). The latter method requires the use of *in vitro* fertilization (IVF) in order to achieve a pregnancy.

The second option is *in vitro* follicular maturation and IVF – a method that has already yielded pregnancies in animal experiments (16). This method is not applicable to the human species, owing to the long period necessary for the primordial follicle to reach the maturation stage (17).

A third method is xenografting of human ovarian tissue into immunodeficient animals (SCID mice) and stimulating it to full follicular maturation (18-20).

To date, it appears that only retransplantation is capable of yielding success. The only pregnancy so far that has resulted in a healthy birth was after transplantation of cryopreserved ovarian tissue into the pelvic wall. The site to which the tissue should be transplanted is a matter of controversy. Orthotopic transplantation may be more physiological but, on the other hand, the tissue is easier to control when it is transplanted heterotopically, *e.g.* under the skin of the arm. One problem after transplantation is neovascularisation of the graft. The

spleen is a very well perfused organ and offers the possibility of easy transplantation, both making the spleen an optimal site of grafting. The present study examined the long-term development of cryopreserved ovarian tissue when transplanted heterotopically under the splenic capsule in ovariectomized rats.

Materials and Methods

Rats. Female rats, 60 days old (Lewis.han inbred strain, LEW/HanHsd), were obtained from Harlan-Winkelmann, Borchen, Germany. The animals were housed in standardized conditions with illumination from 06.00 to 18.00 hours at a room temperature of $22\pm2\,^{\circ}C$. They were fed with standard food for rats (Altromin 1314, Altromin, Lage, Germany) and water *ad libitum*. Groups of five or six rats were housed in one cage (Type 4, Techniplast, Milan, Italy). Approval for the study was obtained from the local ethics committee on animal experiments. The animals were maintained in accordance with Animal Care and Use Committee regulations.

Ovarian tissue. Ovaries from 7-week-old rats were used as donor ovaries. They were obtained by ovariectomy of the female rats under anesthesia with ketamine (80 mg/kg body weight, Ketavet, Pharmacia & Upjohn, Erlangen, Germany) and xylazine (10 mg/kg body weight, Rompun, Bayer, Frankfurt, Germany). Surgery was carried out on a warming plate; the incision site was disinfected with pure alcohol and covered with a single-use sterile towel. Both ovaries were removed *via* a small incision in the abdominal wall, which was sutured with absorbable thread. The ovaries were cleaned of fat and dissected into pieces approximately 1x1x1 mm in size under a stereomicroscope (Leica M 16®, Leica, Bensheim, Germany).

Cryopreservation protocol. The pieces of ovary were equilibrated in ascending equimolar concentrations of dimethylsulfoxide (DMSO)/propanediol up to a concentration of 1.5 M in steps of 0.25 M. The tissue pieces remained in each concentration at $37\,^{\circ}C$ for 7 min and at the last concentration, 1.5 M, for 30 min. The tissue was then placed in 0.3 ml cryovials (CTE, Erlangen, Germany) and loaded into an open freezing system that allows self-seeding (CTE). The freezing protocol was as follows: (a) cool at a rate of $-5\,^{\circ}C$/min to the target temperature of $-3.8\,^{\circ}C$; (b) then cool at $-1\,^{\circ}C$/min to $-5.3\,^{\circ}C$; (c) cool at $-0.2\,^{\circ}C$/min to $-6\,^{\circ}C$; (d) leave temperature ($-6\,^{\circ}C$) unchanged for 20 min; (e) cool at $-0.3\,^{\circ}C$/min to $-30\,^{\circ}C$; (f) cool at $-0.1\,^{\circ}C$/min to $-35\,^{\circ}C$; (g) cool at $-0.3\,^{\circ}C$/min to $-80\,^{\circ}C$; (h) cool at $-10\,^{\circ}C$/min to $-110\,^{\circ}C$; (i) immerse in liquid nitrogen. Seeding occurs automatically at approximately $-5.7\,^{\circ}C$. After storage in liquid nitrogen for at least 1 month, the probes were thawed at room temperature. Removal of the cryoprotectant was carried out in the reverse order of the freezing equilibration procedure. The thawing medium was the same as the freezing medium, with the addition of 0.25 M sucrose. The tissue blocks were then cultured in an antibiotic-supplemented Medicult IVF medium (Medicult, Copenhagen, Denmark) until transplantation.

Transplantation of ovarian tissue. Surgery was carried out under anesthesia with ketamine (80 mg/kg body weight, Ketavet, Pharmacia & Upjohn) and xylazine (10 mg/kg body weight, Rompun, Bayer), irrespective of the stage of the estrus cycle.

During surgery, the rats were kept on a warming plate, and the incision site was disinfected with pure alcohol and covered with a single-use sterile towel. Both ovaries were removed *via* a small incision in the body wall. Transplantation of the cryopreserved ovarian tissue under the splenic capsule was carried out as described earlier (21). Briefly, an incision into the splenic capsule with an 18-gauge needle was used to prepare a pouch into which the ovary tissue pieces (2 per spleen) were carefully placed as deeply as possible. The spleen was relocated into the abdominal cavity, the incision in the muscular part was closed with resorbable thread and the skin was adapted with Prolene or silk 5/0 sutures.

Study design. Seven rats served to provide ovaries for cryopreservation and served as ovariectomized controls (estradiol and uterus weight). Fourteen rats received cryopreserved ovarian tissue and were killed 210 or 300 days after transplantation.

Hormone determination and uterus weight. After the animals had been killed, serum was collected for estradiol measurement by cardiac puncture. Total serum estradiol was measured by RIA without extraction by a commercially available highly sensitive assay for rat and mouse serum (DSL, Sinsheim, Germany). The uterine horns were removed, trimmed and weighed after the surface moisture had been removed.

Tissue examination. The spleen, along with the transplanted ovary or tumor and the uterus, was removed from each rat. The samples were weighed and prepared for histological examination. The tissues were fixed in 5% formalin solution for 3-5 days and subsequently cut macroscopically, dehydrated in alcohol and paraffinized. The paraffin microsections were prepared and stained with hematoxylin-eosin and Gomorri methenamine silver staining.

The size of the tumors was measured at the largest diameter with an accuracy of 0.1 cm.

Statistics. The SPSS® program was used for data evaluation. Nominal data were expressed as mean plus or minus standard deviation (SD) and compared using ANOVA. A *p*-value of 0.05 or less was considered statistically significant.

Results

Tumor incidence, size and histology. All of the recipient animals developed tumors in the spleen. The size of the tumors varied from 0.6 to 1.4 cm. In the group of animals killed after 210 days, the tumor size was 0.9 ± 0.3 cm in diameter (mean$\pm$SD), and in the group of animals killed after 300 days, the tumor size was 1.2 ± 0.2 cm. The differences between the two groups were significant. The tumors showed typical features of mixed granulosa/theca cell tumors. Histologically, the tumor in the spleen was well circumscribed and showed a clear margin from the surrounding lymphoreticular tissue. A faint fibrous capsule was recognizable in some areas. The tumor cells were growing in solid and trabecular sheets, surrounded by fine and sometimes broader bundles of fibrous tissue. They were medium-sized and showed round to oval nuclei with a pale to granular nucleoplasm and clearly recognizable nucleoli,

with a moderately varying size and sometimes abundant cytoplasm. Nuclear grooves or mitoses were rare. No Call-Exner bodies were recognizable. The tumor cells were luteinized over wide areas and showed clearly vacuolated cytoplasm, resembling theca cells. The silver impregnation produced a mixed picture of reticulin fibers surrounding plump tumor trabeculae, alternating with finely dispersed fibers enclosing individual tumor cells. In immunohistochemical staining for alpha-inhibin (mouse anti-human, 1:100, microwave pretreatment; Serotec, Düsseldorf, Germany), the tumor cells showed a distinctive positive reaction (Figure 1).

Estradiol. Estradiol was measured in a highly sensitive estradiol RIA assay for rats. However, the lower detection limit was 5 pg/ml. Sufficient blood serum to measure estradiol was collected from all 14 animals. Estradiol concentrations greater than 5 pg/ml were found in only 3 animals (7.4, 24.3 and 21.3 pg/ml). All of these animals were killed 300 days after transplantation and had large tumors (1.4, 1.3 and 1.2 cm).

Uterus weight. The weight of the uterus in the castrated animals 300 days after castration, in animals with 210-day-old grafts and in the animals with 300-day-old grafts are shown in Table I.

Discussion

Cryopreservation of human ovarian tissue is offered to cancer patients who are facing treatment with gonadotoxic regimens, in order to provide a chance of preserving their fertility. There have been several publications investigating the feasibility of preserving fertility in such patients, and results showing that small follicles in ovarian tissue survive freezing are now no longer controversial (1, 13, 20, 22). Initial attempts to transplant ovarian tissue after cryopreservation were made as early as 1960 by Parrott (23), who was able to obtain live offspring after transplantation of cryopreserved ovarian tissue in mice. Transplantation of ovarian tissue into severe combined immunodeficient mice is now carried out routinely by biomedical researchers (24). There have also been promising results with initial attempts at autotransplantation of cryopreserved ovarian tissue in women (14, 25-28). While Callejo *et al*. (25) and Oktay *et al*. (26) carried out heterotopic transplantation at sites distant from the ovary, Radford *et al*. (27) and Schmidt *et al*. (28) directly transplanted the graft into the stroma of the ovary in women. The main risk of transplanting ovarian tissue in tumor patients is considered to be the potential for disease transmission in gonadal tissue grafts (29). Gonadal tissue grafts may be contaminated with disease and may, therefore, cause a recurrence after reimplantation, as was shown by Shaw *et al*. (30), who demonstrated that lymphoma was

transmitted from affected to unaffected animals by transplantation of ovarian grafts. On the other hand, most malignancies never metastasize to the ovary, so that the risk of reseeding tumor cells should be low; nevertheless, there has been intensive debate on this issue.

Other risks may also arise, such as the potential for malignant transformation of the transplanted ovarian tissue (*e.g.*, due to genetic predisposition) (31).

The present study clearly demonstrated that, after heterotopic transplantation of cryopreserved ovarian tissue under the splenic capsule, the tissue differentiated to form tumors in rats. The histological appearance of these tumors did not differ from that of tumors found after transplantation of fresh ovarian tissue under the splenic capsule (21, 32-35). These tumors had stromal characteristics, with a certain amount of granulosa cells. Sex cord stromal tumors account for approximately 8% of all ovarian tumors, often being associated with endocrine manifestations (36). It is known that the development of these tumors requires a high level of gonadotrophin stimulation (21, 33). The transplantation of ovarian tissue under the splenic capsule caused all estrogens produced by the ovary to drain to the liver. As sex hormone globulin levels are low in adult rats (37), estradiol is metabolized directly and completely during the first passage in the liver, so that no estradiol reaches the pituitary gland to down-regulate gonadotropin secretion. Gonadotrophins, therefore, remain high, as in postmenopausal women or in women who have undergone iatrogenic menopause due to chemotherapy-induced damage to the ovaries. When the tumor reaches a certain size (approximately >0.8 cm), the estrogen production surpasses the metabolic capacity of the liver and estrogenic effects will be detectable (21). In humans, the concentration of sex hormone-binding globulins is high enough to prevent the fast metabolization of estradiol in the liver. Nevertheless, the appearance of sex cord stromal tumors after transplantation of cryopreserved ovarian tissue in this animal tumor model raises the question of what would happen after several years following heterotopic transplantation of ovarian tissues in an organism with high levels of gonadotrophins. In addition, the effect of gonadotrophin stimulation on heterotopically-transplanted tissue is unknown. A third point is the potential for benign sex cord stromal tumors to differentiate and become malignant. It has previously been shown that prolonged gonadotrophin stimulation is capable of causing malignant progression of sex cord stromal tumors in rats (37). Despite the slow growth of granulosa cell tumors in the ovary, they are able to metastasize to the liver and pelvis (38).

This study showed that heterologic autotransplantation of cryopreserved ovarian tissue can result in the growth of sex cord stromal tumors. Although the hormonal situation in rats cannot be completely compared to that in humans, these results may need to be taken into account when
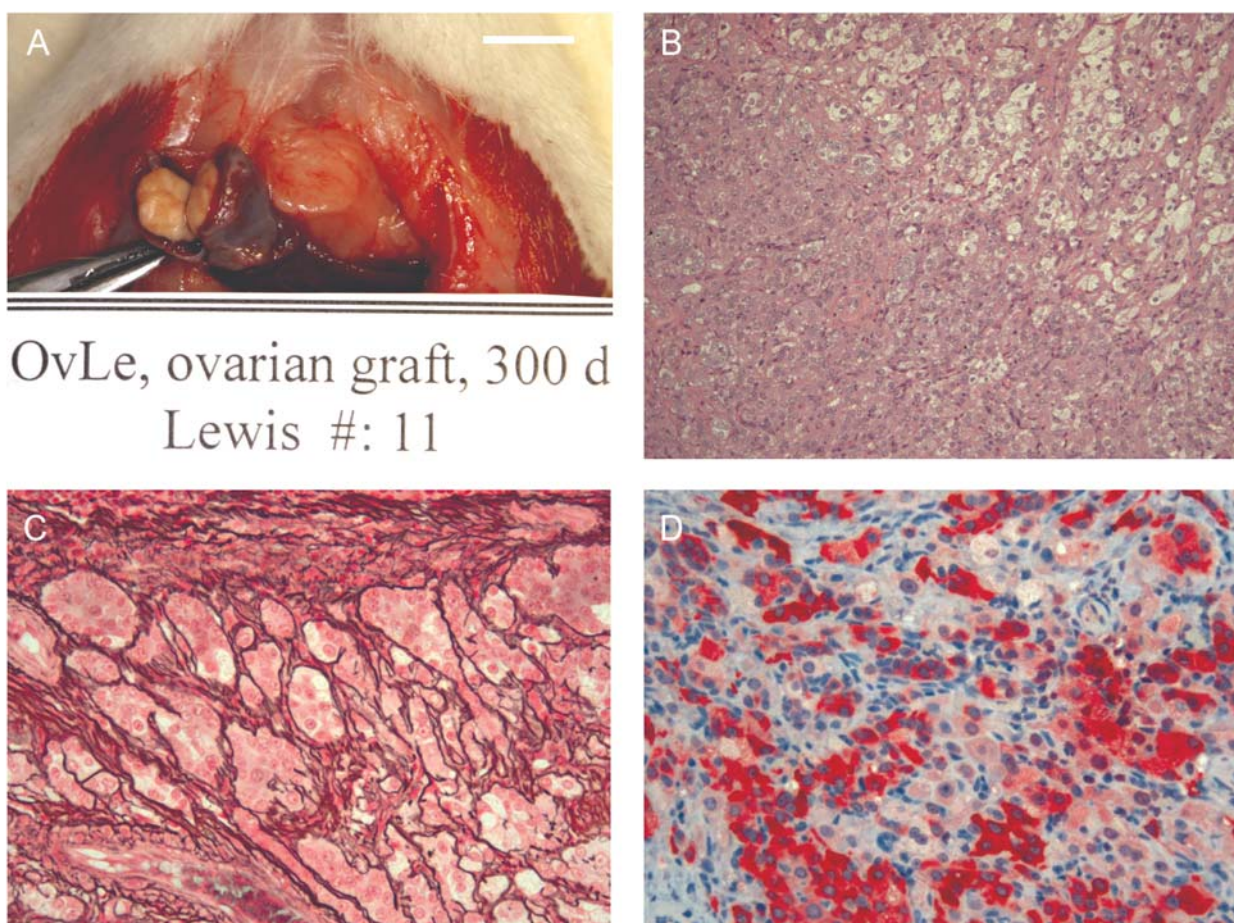
Figure 1. *Sex cord stromal tumors 300 days after transplantation of cryopreserved ovarian tissue. A: in situ (bar indicates 1 cm), B: hematoxylin-eosin stain (original magnification x100), C: silver stain (original magnification x200), D: immunohistochemical staining with inhibin (original magnification x200).*

cryopreserved ovarian tissue is heterotopically transplanted during fertility-preserving programs for cancer patients, who should therefore be carefully observed.

Acknowledgements

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Table I. *Size of tumors after 210 and 300 days of grafting and weight of uteri (mean±SD).*

Group	Castrated without graft	210-day-old grafts	300-day-old grafts
Size of tumors (mm)	-	0.8±0.3[a]	1.2±0.2[a]
Weight of uteri (mg)	187±27[b]	227±71[b]	553±307[b]

mean±SD, n=7, [a,b]statistically significant difference

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